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BACTERIOLOGICAL CRITERIA FOR MARKET OYSTERS

C. B. Kelly

Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio

1960

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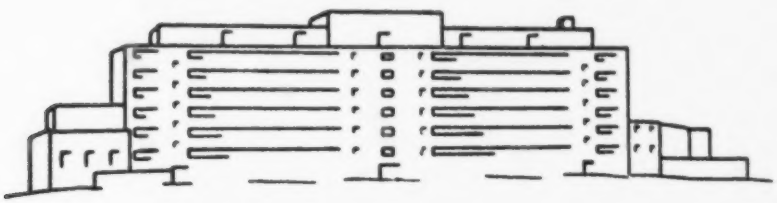

BACTERIOLOGICAL CRITERIA FOR MARKET OYSTERS

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BACTERIOLOGICAL CRITERIA FOR MARKET OYSTERS

A Report on the Results of a Cooperative Study

Prepared for the Participating Agencies

by C. B. KELLY, Chief

Shellfish Sanitation Laboratory

Milk and Food Research Program

INTRODUCTION

The problem of the establishment of safe but reasonable control standards for shellfish as received at the market has been of concern for many years. This problem involves the selection of suitable bacterial indices and numerical limits that will provide a basis for judging whether the food product has been mishandled during processing and shipping, or whether the shellfish were harvested from areas subject to pollution with dangerous human or animal wastes.

The question of bacteriological criteria for market oysters received serious attention at the 1956 Shellfish Sanitation Workship. (1) Consuming and producing States and the Public Health Service presented a collection of data which put serious doubt on the reliability of the bacteriological criteria of the 1946 edition of the Manual of Recommended Practice for the Sanitary Control of the Shellfish Industry. (2) Although the shellfish were harvested from approved areas and processed in adequately equipped and properly operated processing plants, it was not possible in certain areas, particularly during the warmer seasons, to produce shellfish of bacteriological quality in conformity with the criteria stipulated in the 1946 Manual.

The past few years have seen increased interest in the determination of bacteriological quality of shellfish as received at the market. Some agencies established either official or tentative working standards by which they could at least classify the product as received. Possibly the most notable is the classification system established by Canada in 1951, which groups the shellfish into three classes, Acceptable, Acceptable on Condition, and Rejectable, and allows judgment whether a product should enter or be tolerated in the consuming area. The Acceptable-on-Condition classification has the added advantage of encouraging investigation by the producing State of plants producing a mediocre product, thus detecting and eliminating somewhat unsatisfactory conditions and effecting an over-all improvement in quality of the product. Because of the success of Canada with this scheme, the method was accepted in principle at the Shellfish Sanitation Workshop, with the modification of extending the coliform MPN limit in the Acceptable class from 2,400 to 16,000. The bacteriological criteria established at the 1956 Shellfish Sanitation Workshop are as follows:

Class 1. Acceptable:

Shucked oysters with a Most Probable Number (MPN) of coliform bacteria of not more than 16,000 per 100 ml., and/or a Standard Plate Count of not more than 50,000 per ml.

Class 2. Acceptable on Condition:

Shucked oysters with a coliform MPN greater than 16,000 per 100 ml. but less than 160,000 per 100 ml., and/or a Standard Plate Count greater than 50,000 per ml. but less than 1,000,000 per ml.

(The oysters will be accepted on the condition that the shellfish sanitation authority in the originating State will make im-

mediate investigations of the producer's plant and operations, and will submit a report of such investigations to the control agency in the market area. On the basis of this report the control agency in the market area will reject or permit further shipments from the producer in question.)

Class 3. Rejectable:

Shucked oysters with a coliform MPN of 160,000 or more per 100 ml., and/or a Standard Plate Count of 1,000,000 or more per ml.

It was recommended at the Workshop that these criteria be applied for a two-year interim period by the shellfish control agencies in the receiving areas. It was proposed that during this period studies would be conducted to evaluate the applicability of the tentative standards. It was also recommended that serious study be made of the validity of the coliform group and the plate count as indices of bacteriological condition of market oysters, and further that investigations be initiated to determine the validity of other bacterial indices. E. coli was suggested as the organism which should receive the most attention.

PLAN OF STUDY

A short time later, representatives of State, Federal, and municipal control agencies met in Washington, D.C. to outline a plan of study to evaluate these market criteria. The group included representation from Maryland, Virginia, New Jersey, New York State, New York City, and the shellfish control and research sections of the Public Health Service. The plan of investigation formulated by that group involved the collection and examination of oysters from representative shucking and repacking establishments by the control agencies in the producing area and of shipments from these same sources by shellfish agencies in the consuming area. Examinations were to be conducted for coliform MPN, plate count, and E. coli MPN, following procedures which were agreed to and outlined by the Shellfish Sanitation Laboratory. A copy of these procedures is incorporated in the Appendix of this report.

This study proceeded throughout almost two complete oyster harvesting seasons. The results obtained by all of the cooperating agencies were collected and summarized by the Public Health Service, Shellfish Sanitation Laboratory, following procedures recommended by Dr. E.K. Harris, Chief, Statistical Services, Robert A. Taft Sanitary Engineering Center. The results of this study were considered by the participating agencies at a meeting in Washington, D.C. on June 26, 1958.

CONFORMITY WITH TENTATIVE MARKET STANDARDS

The States of Maryland and Virginia examined more than 300 samples of oysters at various stages of harvesting, shucking, and repacking. Of these samples, the data collected by the State of Virginia included 95 sets of samples of shucked oysters as packed in Virginia shucking houses. The data collected by the State of Maryland included 56 samples of shucked oysters as received from Virginia by Maryland repacking houses and 37 samples of these oysters after blending, washing, and repacking. New York City presented data on the examination of 39 samples received from Maryland repacking establishments, but, in all, New York City submitted results of the examination of almost 300 samples of shucked oysters as received at the New York market, 196 from Virginia and Maryland sources and 98 from New Jersey, Delaware, and Connecticut. In addition, the New York State Conservation Department submitted results of the examination of 27 samples shipped by two Maryland repackers. The Food and Drug Directorate of the Canadian Department of National Health and Welfare submitted results on 66 samples of shucked oysters examined on receipt in the Canadian market.

Although not included in this report, additional data were presented at the 1958 Shellfish Sanitation Workshop. (3) The Chicago Board of Health presented the results of the examination of 360 samples of shellfish collected in the Chicago markets, and the Louisiana State Department of Health presented a summary and analysis of bacteriological results obtained on samples of shell and shucked oysters collected from growing areas and in shellfish shucking houses.

All of the samples collected at the same stage in processing and marketing were submitted to a summarization procedure which utilizes log probability plots in accordance with the method first described by Velz (4) and later by Thomas (5). Preliminary frequency distributions of the data on a year-round basis indicated extreme variation. Sorting the data by months revealed that higher values were obtained during the months of April, September, October, and November than during the months of December, January, February, and March. Therefore, when the number of samples allowed subdivision into these two seasonal groups, separate probability plots were prepared.

Coliform MPN's and plate counts of oysters at three probability percentiles are shown in Tables 1 and 2. Values are shown for two selected seasons as well as for the entire harvesting period. These two tables show primarily the bacterial changes taking place during processing and shipment. It can be noted that in all instances, a definite seasonal variation was found. Both the coliform MPN and the plate counts were significantly higher during the warmer periods of the oyster harvesting season than during the colder months. It can also be noted that significant increase took place not only during handling but also during shipment, the latter most likely due to deficiencies in refrigeration.

The data contained in Tables 3 and 4 illustrate the extent of compliance with limits for coliform MPN and plate count suggested at the 1956 Shellfish Sanitation Workshop. The low percent attainment of suggested limits at the various stages of processing raises some question as to the reasonableness of the current tentative criteria of quality. MPN 230 was included primarily to illustrate the degree of conformity of the shell stock with the limit suggested in the revision of Part 1 of the Public Health Service Manual of Recommended Practice For Sanitary Control of The Shellfish Industry but has much less significance when applied to shucked oysters. Plate counts of 5,000 and 10,000 were included to show the degree of conformity with these arbitrarily selected limits and give some idea of the range of the plate count obtained at various stages of processing and marketing.

The data indicate a high degree of compliance with the recommended market standards during the colder months. Eighty percent of the Maryland and Virginia oysters as received at

TABLE 1. COLIFORM MPN AT 10, 50, and 90 PROBABILITY PERCENTILES FOR SHELL AND SHUCKED OYSERTS

	Shucking		Maryland repacking		New Jersey repacking		Market		
Percentile	Shell stock	As packed	As received	As repacked	As received	As repacked	Maryland repacker	All Md. & Va. packers	Packers N. of Md.
				Dec Jan Feb Mar					
10	18	150	940	480	320	40	1,100	160	<18
50	240	1,400	7,200	5,000	6,600	520	9,200	2,800	130
90	3,800	14,000	54,000	52,000	13,000	8,000	75,000	50,000	22,000
No. of samples	39	50	38	25	25	23	31	139	93
				Apr Sept Oct Nov					
10	100	920	2,400	1,500	----	---	----	2,000	80
50	2,000	11,000	19,000	6,400	----	---	----	13,000	3,500
90	35,000	110,000	140,000	26,000	----	---	----	80,000	140,000
No. of samples	21	45	18	12	----	---	----	66	26
				All Seasons					
10	16	270	1,100	800	----	---	1,700	230	<18
50	490	3,500	10,000	5,300	----	---	11,000	4,100	390
90	15,000	44,000	90,000	35,000	----	---	76,000	74,000	39,000
No. of samples	60	95	56	37	----	---	39	205	119

TABLE 2. STANDARD PLATE COUNTS AT 10, 50 and 90 PERCENTILES FOR SHELL AND SHUCKED OYSTERS

Percentile	Shucking		Maryland repacking		Market		
	Shell stock	As packed	As received	As repacked	Maryland repackers	All Md. & Va. packers	Packers N. of Md.
			Dec Jan Feb Mar				
10	100	960	2,600	<3,000	1,900	1,400	800
50	800	5,500	14,000	4,900	27,000	15,000	7,000
90	6,700	31,000	76,000	100,000	260,000	160,000	150,000
No. of samples	34	50	38	25	30	99	99
			Apr Sept Oct Nov				
10	140	1,800	8,600	---	---	2,600	1,000
50	2,500	17,000	36,000	---	---	22,000	7,000
90	45,000	150,000	140,000	---	---	190,000	170,000
No. of samples	20	45	18	18	8	98	27
			All Seasons				
10	120	1,100	3,200	1,100	4,000	1,500	900
50	1,200	7,600	19,000	8,600	30,000	16,000	7,000
90	12,000	70,000	110,000	70,000	220,000	170,000	160,000
No. of samples	54	95	56	37	38	197	126

TABLE 3. PERCENT ATTAINMENT OF MPN LIMITS FOR SHELL AND SHUCKED OYSTERS

MPN	Shucking		Maryland repacking		New Jersey repacking		Market		
	Shell stock	As packed	As received	As repacked	As received	As repacked	Maryland repacker	All Md. & Va. packers	Packers N. of Md.
				Dec Jan Feb Mar					
230	48	15	----	----	--	----	--	---	---
2,400	86	62	24	34	72	72	20	48	75
16,000	97	91	69	74	92	95	63	80	90
<160,000	99.9	99.7	97.5	96	99	99.9	96	97	97
No. of samples	39	50	38	25	25	23	31	139	93
				Apr Sept Oct Nov					
230	18	2	----	----	--	----	--	---	---
2,400	54	21	10	20	--	----	11	12	44
16,000	82	59	47	80	--	----	47	56	80
<160,000	97.5	92	91	99.9	--	----	91	96	91
No. of samples	21	45	18	12	--	----	8	66	26
				All Seasons					
230	39	8	----	----	--	----	--	---	---
2,400	72	42	20	29	--	----	15	40	70
16,000	91	78	61	77	--	----	60	72	85
<160,000	98.5	97	95	99	--	----	96	95	96
No. of samples	60	95	56	37	--	----	39	205	119

TABLE 4. PERCENT ATTAINMENT OF PLATE COUNT LIMITS, SHELL AND SHUCKED OYSTERS

Standard plate count	Shucking		Maryland repacking		Market		
	Shell stock	As packed	As received	As repacked	Maryland repackers	All Md. & Va. packers	Packers N. of Md.
			Dec Jan Feb Mar				
5,000	87	46	----	----	----	---	---
10,000	94	68	40	----	28	40	56
50,000	99.4	95	83	----	64	74	77
500,000	----	99.9	----	----	96	97	96
<1,000,000	----	----	99.9	----	98	99	98
No. of samples	34	50	38	----	30	139	99
			Apr Sept Oct Nov				
5,000	62	24	----	----	----	---	---
10,000	73	38	12	----	----	32	57
50,000	91	74	62	----	----	70	78
500,000	99.1	98	----	----	----	97	96
<1,000,000	----	99.9	99.9	----	----	99	98
No. of samples	20	45	18	----	8	139	27
			All Seasons				
5,000	78	37	----	----	----	---	---
10,000	88	54	32	54	25	40	56
50,000	98	86	77	86	63	72	77
500,000	----	----	99.2	99.5	96	97	96
<1,000,000	----	----	99.8	99.8	98.6	99	98
No. of samples	54	90	56	37	38	205	126

the markets were within the acceptable class as judged by either coliform MPN or plate count, and another seventeed percent were acceptable on condition. During the warmer periods compliance was much lower, particularly in the acceptable class. Only 56 percent of the Maryland and Virginia oysters were within coliform MPN 16,000, the upper limit of the 1956 "Acceptable on Condition" class.

The influence of temperature can also be seen by comparison of the data for Maryland and Virginia oysters with those from more northern sources. In both seasons the percentage compliance with coliform limits is much higher in the northern oysters. The effect of temperature can also be seen in the lower percentage compliance in northern oysters during the warmer period.

RELATIONSHIPS BETWEEN THE COLIFORM GROUP AND E. COLI

The bacteriological data presented at the 1956 Shellfish Sanitation Workshop suggested very strongly that the coliform group was not an entirely staisfactory index of quality of shucked oysters at the market level. The tolerances for coliform MPN's were accordingly relaxed in the tentative market standards. In many of the presentations at the Workshop there appeared the further suggestion that a frank evaluation be made of the relative significance of the coliform group and E. coli as measures of sanitary condition of shucked oysters. Therefore, the agencies cooperating in this study decided to include the determination of E. coli MPN in the program of evaluation of the tentative market standards.

The first stage in the investigation of the significance of E. coli was the preparation of a set of procedures for the enumeration of these organisms. There are no standard methods for this test, even in such publications as Standard Methods for the Examination of Water, Sewage and Industrial Waste (6) or in the Recommended Procedures for the Bacteriological Examination of Shellfish Waters and Shellfish. (7) Lack of uniformity in procedures is probably one of the

TABLE 5. COLIFORM, E.C. GAS POSITIVE, AND E.C. COMPLETED TEST MPN'S, SHUCKED OYSTERS AT VARIOUS STATES OF PROCESSING

Stages in Process	Coliform		E. C. Gas Positive		E. C. Completed	
	50%	90%	50%	90%	50%	90%
As packed						
All year	700	19,000	<18	170	<18	150
As received						
Summer	20,000	120,000	63	1,000	27	360
Winter	5,500	66,000	<18	130	<18	88
All year	7,200	66,000	<18	300	<18	190
As repacked						
All year	4,600	27,000	<18	320	<18	120
Market						
New York City						
Md. and Va.						
Summer	16,000	160,000	30	1,100	<18	660
Winter	6,800	90,000	<18	100	<18	50
All year	11,000	120,000	<18	330	<18	230
New York State						
2 Md. Packers	2,600	21,000	<18	90	<18	24
All sources	1,400	18,000	<18	240	<18	94
Canada						
All samples						
North & South	3,500	400,000	<18	360	<18	300
New York City						
North of Md.						
All year	2,800	68,000	<18	490	<18	350

most important hindrances to acceptance of tests for E. coli as an index of sanitary quality. The group therefore developed a procedure to be followed in this study.

The procedure is a liquid confirmatory method applied to all gas positive presumptive broth tubes, using Hajna's and Perry (8) E.C. medium incubated at elevated temperatures. The details are given in the Appendix. Recognizing the lack of information concerning the reliability of the procedure, the plan included an evaluation applying the completed-IMViC test on the presumptive tubes from which the E.C. tubes were transferred.

A summary of the results obtained is presented in Table 5, arranged in somewhat the same form as the coliform MPN and plate count data in Tables 1 and 2. Coliform MPN's and E.C. Medium gas positive and E.C. completed test MPN's are compared at two probability percentiles. MPN's based on the presence or absence of gas in the E.C. tube are presented to compare the partially confirmed test with the completed test results.

Seasonal variations in E.C. gas positive MPN and E. coli completed test MPN were not as great as in coliform MPN's. To some extent the seasonal increases E.C. test MPN's may be the result of bacterial multiplication, but at least part may be attributed to increased activity of the oysters in the warmer waters of the summer season.

A comparison of the E. coli MPN's, both gas positive and completed test, shows little change on passage through the handling and shipping processes.

TABLE 6. E.C. GAS POSITIVE AND E. COLI COMPLETED MPN'S AT 90 PERCENTILE - ALL SEASONS

As packed	As received at repackers	As repacked	New York City	New York State	Canada
E.C. Gas Positive					
170	300	320	330	90	360
E. coli completed					
150	190	120	230	24	300

TABLE 7. PERCENT ATTAINMENT OF E.C. GAS POSITIVE AND E. COLI COMPLETED TEST MPN'S

MPN	Repacker			Market			
				New York City		N.Y.	
	As packed	As received	As repacked	Md. & Va.	N. of Md.	State	Canada
			E.C. Gas Positive				
<18	59	54	47	50	49	46	43
20	78	60	53	66	62	68	57
230	94	88	87	87	84	96	86
1,600	96	97	97	95	94	99+	97
			E. coli Completed				
<18	63	59	63	62	65	86	50
20	84	69	71	75	69	90	63
230	94	92	94	90	88	96	89
1,600	96	98	98	96	96	99+	97

Table 7 shows the frequency of occurrence at the different stages in processing and shipment of four arbitrarily selected MPN values. A characteristic of the E. coli results in the high frequency of very low or indeterminate values. For example, 62 percent of the samples of Maryland and Virginia oysters sampled by New York City showed E. coli completed test MPN's of less than 18 and 75 percent showed MPN's of 20 or less. Two-thirds of these samples showed E.C. gas positive MPN's of 20 or less.

The data in Table 7 also show that 88 to 96 percent of all samples of shucked oysters at the market had an E. coli completed test MPN of 230 or less and 84 to 96 percent of the same samples had an E.C. gas positive MPN of 230 or less.

RELATIONSHIPS BETWEEN E.C. GAS POSITIVE AND E. COLI COMPLETED TESTS

The E.C. completed test, because it involves classification by IMViC, is extremely demanding of time and materials. Minimum time for obtaining a completed test result is nine days after the initial inoculation in the presumptive broth. If the partially confirmed test E.C. test could be applied with reasonable reliability, results would be available in three days most of the time, or occasionally in four to five days. The E.C. liquid confirmatory procedure is no more involved than the liquid confirmatory test for coliform organisms.

A comparison of the E.C. gas positive E.C. completed test MPN's shown in Table 7 shows that the disagreement between these two methods is chiefly in the lower MPN ranges. To what extent this is a true relationship and not experimental error, both in the test and in the method of enumeration, is difficult to determine without basic experimentation, but the data show a gradually decreasing difference between E.C. gas positive and E.C. completed test on approach to MPN 230. At that level, the difference in the percent occurrences of that MPN by E.C. gas positive and E. coli completed tests in market oysters differed by only 3, 4, 0 and 3 in Table 7.

To demonstrate the variations obtained in E.C. gas positive MPN's at the lower levels, Table 8 presents the frequency of findings compared to E. coli completed MPN 20 or less.

TABLE 8. E.C. GAS POSITIVE MPN'S OBTAINED AT E. COLI COMPLETED TEST MPN 20

E.C. Gas Positive MPN	Number of Samples						Percent	
	Va.	Md.	State	City	Canada	Total	Actual	Accumulated
<18	32	37	13	60	13	155	66.5	66.5
20	11	11	5	20	3	50	21.5	88.0
45	3	2	4	5	1	15	6.4	94.4
78			1	5		6	2.5	96.9
130			1	4		5	2.1	99.0
230					1	1	0.4	99.4
330					1	1	0.4	99.8
2,400				1		1	0.4	100
Total	46	50	24	95	19	234		

The data show that at E. coli completed MPN 20 or less almost 95 percent of the E.C. gas positive results were in agreement if a latitude of two tubes is allowed, and only 1% of the samples showed gas positive above MPN's 130 when the completed test MPN was 20 or less.

FALSE POSITIVE E.C. PARTIALLY CONFIRMED TESTS

In the E. coli procedure evaluation, the false positive E.C. partially confirmed tests Type I were revealed by the failure to recover E. coli Type 1 in the completed - IMViC test performed on the gas positive E.C. tubes.

By far the most frequent IMViC coliform type responsible for the false positive tests in the cooperative study was Aerogenes type 1 (IMViC type --++). Next in order of occurrence, but not necessarily in order of ability to produce gas in E.C. medium was E. coli type 2 (IMViC -+--.). Stuart et al (9) in investigations of the Eijkman reaction of coliform organisms, found that these two were the only coliform types other than E. coli type 1 showing ability to produce gas in Eijkman broth in a water bath at 45.5° C. Stuart found that about 2 percent of 680 cultures of IMViC type --++ produced gas, and about 85 percent of 134 IMViC type -+-- cultures produced gas at 45.5° C. In the work at the Sanitary Engineering Center (9) 10.6 percent of the IMViC type --++ and 21.8 percent of the IMViC type -+-- produced gas in E.C. medium at 45° C.

From the data available to him at the time, Dr. E.C. Harris has computed the "apparent percent false positive" E.C. partially confirmed tests. Values of 27, 39 and 50 percent were obtained on data also submitted by three of the participating agencies. Dr. Harris compared these with computed values of 33 percent, 34 percent and 8 percent for data collected on the Gulf Coast.

These variations in percentage false positives are not unique to this study. Investigations at the Sanitary Engineering Center (10) on cultures of coliforms received from 14 geographical areas have shown a definite variation, associated with geographical area in the ability of coliforms other than E. coli type 1 to produce gas in E.C. medium at 45.0° C. Similar behavior was found on the use of boric acid lactose broth. (11)

The specificity and constancy of the test are also greatly affected by temperature of incubation. All investigators of the test have established very close tolerance, usually + 0.2° C, for temperature of incubation. The procedures for this study had detailed stipulations for temperature and condition of incubation. Reduction of incubation temperature by as little as 0.5° C has been found by participants in this project to have caused significant differences in the numbers of false positive tubes.

DISCUSSION

In a cooperative study undertaken by nine shellfish agencies samples of oysters have been examined for coliform MPN, E.C. gas positive MPN, E. coli completed test MPN and standard plate count to evaluate (a) the applicability of the tentative market standards for shucked oysters adopted at the 1956 Shellfish Sanitation Workshop and (b) the relative significance of the coliform group and E. coli as measures of sanitary condition of market shucked oysters. The study included the examination of samples of shell and shucked oysters at all stages of handling and shipment from the shell stock before shucking to the final product as received in the consuming areas. The project continued throughout two complete shellfish harvesting seasons.

The results obtained in this cooperative study were discussed at a meeting held on June 26, 1958. After a review of a detailed summary it was recommended that a report on the study be prepared for presentation at the 1958 Shellfish Sanitation Workshop.

The results revealed a high degree of compliance with the coliform MPN stipulations of the tentative market standards in all sections of the study area, although definite seasonal and geographical differences were observed. Results on shucked oysters from sources in Maryland and further south showed considerably higher coliform MPN's than those from the more northern areas. This would suggest that limits based on coliform MPN are unrealistic because they exaggerate seasonal and geographical differences.

Compliance with the present plate count limits was consistent in oysters obtained from all areas. There was an indication, however, that the plate count upper limit of one million

for the "Acceptable on Condition" class is too liberal and should be reduced to 500,000.

MPN's obtained by both E.C. gas positive test and the E. coli completed test show considerably less variation than coliform MPN's in oysters at all stages of processing and marketing. As an index of pollution of fecal contamination, therefore, the first two tests would be the more useful, once the reliability of the procedure and inherent experimental errors are more fully determined.

The E.C. results, both gas positive MPN and completed test MPN, are characterized by a high percentage of low or indeterminate values. Moreover, these two tests differ most at low MPN values. These findings suggest an appropriate latitude must be allowed in setting the limits for assessment of quality. Sample-to-sample comparisons of MPN's obtained have shown that these two methods agree within 3 positive tubes.

Preliminary estimates have been obtained of the reliability of the E.C. partially confirmed test as an index of the presence or absence of E. coli. "Apparent False Positive Percentages" computed for the data submitted by individual laboratories show some variation from one laboratory to another.

After consideration of the summaries of the results obtained, the group which met in Washington on June 26, 1958 arrived at the following conclusions and recommendations:

1. Bacteriological criteria of sanitary quality serve a useful purpose as applied to shucked oysters in the market.

2. The standard plate count serves as a useful index of unsatisfactory handling during processing, improper refrigeration, or mishandling while the product is in transit.

3. The coliform MPN is not much more meaningful than the standard count as a criterion of quality.

4. Serious consideration should be given to the substitution of E. coli for the coliform group as an index of fecal contamination of shucked oysters.

ACTION AT 1958 SHELLFISH SANITATION WORKSHOP

After this report was presented at the 1958 Shellfish Sanitation Workshop, the group recommended to adopt in principle the conclusions of the report. On that basis, the following decisions were made:

1. The entire coliform group of organisms is not a meaningful indicator of pollution in shucked oysters at the market. The Workshop recommended the adoption of a new interim standard based on densities of "fecal coliform organisms" as indicated by a partially confirmed test using E.C. medium performed on all positive presumptive tubes, according to the procedures recommended for the 1956-58 cooperative study.

2. The plate count should be included as an index of contamination during processing or of faulty handling or refrigeration practices during transportation and marketing.

3. The sanitary significance of "fecal coliform organisms" cannot properly be established without reliable information on the origin and introduction into shellfish growing areas. The Workshop therefore recommended that studies be conducted in shellfish growing areas to determine the significance of these organisms in shellfish growing areas and requested the Public Health Service to develop a uniform procedure for sanitary survey in areas of known pollution to be followed by the States in conducting such investigations. Preliminary results are to be discussed at the 1960 Shellfish Sanitation Workshop.

4. The following bacteriological standards for shucked Eastern oysters at the market level were adopted for use in the Cooperative Program for the Certification of Interstate Shellfish Shippers during 1959 and 1960:

Satisfactory:

A fecal coliform density (MPN) of not more than 78 per 100 ml. of sample, and a plate count of not more than 100,000 per ml. of sample, except that a fecal coliform density up to and including 230 per 100 ml. of sample and/or plate count up to and including 500,000 per ml. of sample will be acceptable in occasional

samples. (For convenience, these will be referred to as Class I-A and I-B samples, respectively.)

The official agency in the receiving area should notify both the shipper and shellfish control agency at the point of production of any Class I-B results. If two consecutive Class I-B shipments are found, the receiving area is justified in excluding the shipper from the market area until a satisfactory report on the shipper is received from the State control agency in the producing area.

Unsatisfactory:

A fecal coliform density (MPN) of more than 230 per 100 ml. of sample or a plate count of more than 500,000 per ml. of sample. (For convenience, these will be referred to as Class II samples.)

The official agency in the receiving area should immediately notify both the shipper and shellfish control agency in the producing area of a Class II result. A single Class II result is justification for excluding the shipper from the market pending receipt of a satisfactory explanation from the official control agency in the producing area.

For the purpose of use in the Interim Bacteriological Standard, the term fecal coliform organisms is defined as those organisms which, on transfer to E.C. medium from gas positive presumptive broth tubes, show the production of gas after incubation for 48 hours at 44.5° C. in a water bath.

The procedure as described in the Appendix recognizes air incubation at 45.5° C + 0.2° C as an alternate to incubation in a water bath at 44.5° C + 0.2° C. Air incubation is acceptable only provided that a uniform lag period and temperature control are attained throughout the incubator under all conditions of loading.

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APPENDIX

PROCEDURES FOR THE BACTERIOLOGICAL EXAMINATION OF OYSTERS

PROCEDURES FOR THE BACTERIOLOGICAL EXAMINATION OF OYSTERS

I. Collection and Transportation of Samples

Samples of oysters should be examined within six hours after collection and in no case shall they be examined if they have been held more than 24 hours. The report of examination shall include a record of the time elapsed between collection and examination.

A. Shell Stock

In general, twelve or more oysters judged to be representative of the lot to be sampled shall be selected. The oysters selected shall have unbroken lips and the shells tightly closed. The number of oysters collected shall be sufficient to produce not less than 200 grams of meats and shell liquor.

Samples of oysters shall be collected in sterilized containers. Suitable containers for the purpose are a can with lid or a waterproof bag. The samples shall be kept in dry storage at a temperature between 0° C. and 10° C. until examined.

B. Shucked Oysters

Samples of shucked oysters may be taken in the original container, if of suitable size; or the oysters may be transferred to a sterile wide mouth jar, using sterile forceps or spoon. Not less than 200 grams (approximately 1/2 pint) shall be taken as a sample. The sample shall be refrigerated immediately after collection by packing in crushed ice and kept in crushed ice until examined.

C. Frozen Shucked Oysters

If the package contains 16 ounces or less, one or two packages may be taken as a sample. Samples from larger blocks may be taken by coring with a suitable instrument or by quartering, using sterile technic. The sample shall be 200 grams or more. Cores or quartered samples shall be transferred to sterile wide mouth jars for transportation to the laboratory.

II. Preparation of Sample for Examination

A. Shell Oysters

1. Cleaning the Shells - Before starting to clean the shells, the hands of the examiner shall be thoroughly scrubbed with soap and water.

Scrape off all growth and loose material from the shell and scrub the oysters with a sterile stiff brush under running water, paying particular attention to the crevices at the junctions of the shells. The water shall be from a supply approved by the appropriate state agency as a drinking water supply. Place the cleaned oysters in clean containers or on clean towels and allow to drain in the air.

2. Removal of Shell Contents - Before starting the removal of shell contents, the hands of the examiner shall be thoroughly scrubbed with soap and water and rinsed with 70 per cent alcohol. Hold the oyster in the left hand, with the deep shell on the bottom and the hinge pointed away from the examiner. Using a sterile oyster knife, insert the point between the shells on the ventral side (at the right of the

examiner) about one-fourth the distance from the hinge to the bill. Entry may also be made at the bill after making a small opening with an instrument similar to bone cutting forceps, suitably sterilized.

Cut the adductor muscle from the upper flat shell and pry the shell wide enough to drain the shell liquor into a sterile tared beaker or wide mouth jar. The upper shell may then be pried loose at the hinge, discarded, and the meats then transferred to the beaker or jar after severing the muscle attachment to the lower shell.

Open a sufficient number of oysters to obtain approximately 200 grams of meats and shell liquor, except that not less than six oysters shall constitute a sample.

B. Shucked Oysters

Transfer approximately 200 grams of oysters from the sample jar to a sterile tared beaker or jar, using sterile forceps or spoon.

C. Dilution and Grinding

Weigh the sample to the nearest gram.

Transfer the weighed sample to a sterile grinder and add an equal amount by weight of sterile phosphate buffered dilution water.

Grind for 90 seconds in a blender similar to a Waring Blendor operating at 14,000 RPM.

III. Examination for Members of the Coliform Group

A. Inoculation

The ground sample should be inoculated immediately. If there is any delay, it shall be thoroughly mixed immediately before innoculation.

Lauryl tryptose broth, single strength in fermentation tubes, shall be used as the presumptive test medium. Dilutions shall be made in sterile phosphate buffered dilution water.

Inoculate:

1. Each of 5 tubes with 2 ml. of prepared sample. (Equal to 1 gram of oysters.)
2. Each of 5 tubes with one ml. of a 2 to 8 dilution of prepared sample. (Equal to 0.1 gram of oysters)
3. Each of 5 tubes with one ml. of a 1 to 9 dilution of the dilution prepared in (2) above. (Equal to 0.01 gram of oysters)
4. Each of 5 tubes with one ml. of a 1 to 9 dilution of the dilution prepared in (3) above or one-tenth ml. of the dilution prepared in (3). (Equal to 0.001 gram of oysters.)

In order to avoid indeterminate results, extension of the dilutions suggested above may be necessary. The amounts of sample selected for inoculation should be such that the largest portions will give positive results in all or the majority of tubes and the smallest portions will give negative results in all or the majority of tubes. To attain this with any degree of assurance with samples of doubtful quality it will be necessary to inoculate four decimal dilutions.

B. Presumptive Test

Incubate the fermentation tubes at 35° C + 0.5° C. Examine each tube at the end of 24 ± 2 hours and, if no gas has formed, again at the end of 48 + 3 hours. Formation of gas may be recognized by the presence of visible gas in the upper end of the inverted fermentation tube or the presence of gas bubbles throughout the medium outside the fermentation tube when gently shaken.

Formation of gas within the stipulated incubation periods constitutes a Positive Presumptive Test which must be confirmed:

The absence of gas formation after 48 hours incubation constitutes a Negative Test for coliform group organisms.

C. Confirmed Test for Coliform Organisms

All presumptive test fermentation tubes positive at the end of 24 hours or 48 hours incubation shall be subjected to the confirmed test. If active fermentation appears in the primary fermentation tube before the expiration of the 24 hour period it is preferable to transfer these cultures without waiting for the completion of the full period.

In routine examinations, where the proportion of false positive presumptive tubes is known to be low, confirming all tubes may not be necessary. Sufficiently accurate results may be obtained if confirmation is conducted on the tubes of only the two highest dilutions showing gas when all five tubes of the lower dilution are gas positive. However, in the event of "skips", confirmation should be conducted on all tubes of all dilutions showing less than five tubes positive.

1. Transfer a loopful (loop not less than 3mm diameter) of culture from the presumptive tube to a fermentation tube containing brilliant green lactose bile broth.

2. Incubate the inoculated tubes for 48 $\pm$ 3 hours at 35° $\pm$ 0.5° C.

3. Formation and presence of gas in any amount in the inverted vial of the confirmatory tube within 48 hours constitute a Confirmed Test for coliform organisms.

D. Expression of Results

The coliform group density shall be expressed as "Most Probable Number" (MPN) per 100 grams of sample. MPN tables and a discussion of the method of computing the MPN will be found in Standard Methods for the Examination of Water, Sewage, and Industrial Wastes, Tenth Edition pp 382-387.

IV. Standard Plate Count

A. Plating

In preparing the plates, such amounts of sample should be planted as will give from 30 to 300 colonies per plate and the aim should be to have at least two plates giving colonies between these limits.

1. Add the appropriate amount of sample, using the dilutions prepared for the inoculation of the presumptive tubes, to the petri dish. Usually 0.1 and 0.01 gram amounts of shell and shucked oyster samples taken in shucking plants and 0.01 and 0.001 gram amounts of shucked oysters sampled in the market will produce plates of the desired density.

2. Add not less than 10 ml. of liquified tryptone glucose extract agar at a temperature of 43° to 45° C. to the inoculated dish. Not more than 20 minutes should elapse between plating and pouring. Mix the medium and sample by tilting and rotating the dish.

3. Incubate the solidified plates at 35° $\pm$ 0.5° C. for 48 hours.

B. Counting

Counting shall be done with an approved counting aid, such as the Quebec Colony Counter.

C. Expression of Results

The count shall be expressed as Standard Plate Count per gram of sample. The procedure for computing the Standard Plate Count as well as complete details concerning the entire Standard Plate Count procedure will be found in Standard Methods for the Examination of Water, Sewage, and Industrial Wastes, Tenth Edition, pp 373-374, and in Standard Methods for the Examination of Dairy Products, Tenth Edition, pp 90-113.

V. Liquid Confirmatory Procedure for E. coli and "Fecal Coliform Organisms"

The relative density of E. coli and "fecal coliform organisms" in both plant and market samples being unknown but most likely much lower than that of the coliform group will require submitting all gas positive presumptive tubes to the liquid confirmatory procedure for E. coli and "fecal coliform organisms". Therefore, until the procedure has been evaluated and the coliform relationships have been determined, the scheme of eliminating certain of the gas positive tubes in the lower dilutions as outlined in the confirmed test for coliform organisms (III-C of the Recommended Procedures) shall not be followed in this test for E. coli.

In view of the many divergent opinions concerning the significance of gas production in the confirmatory media as an indication of the presence or absence of E. coli, confirmatory procedures are recommended until experience has demonstrated the reliability of the procedure. All gas positive liquid confirmatory tubes shall be submitted to a completed-IMViC procedure as shown in the attached outline. To determine the number of false negative reactions in the liquid confirmatory broth the procedure rec-

ommends parallel completed-IMViC tests at 35° C. conducted directly from the gas positive presumptive broth tubes.

The procedure recognized two methods of incubation: water bath at 44.5° C ± 0.2° C and air incubation at 45.5° C ± 0.2° C. Tests have demonstrated that water bath and air bath incubation produce different patterns of temperature attainment in the culture medium. The least lag in attainment of temperature was accomplished in a water bath equipped with a stirring device.

In view of the evidence that air incubation at 45.5° C in a well operated air incubator will produce acceptable results, this method is recognized as an alternate provided that a uniform lag period and temperature control are possible throughout the incubator under all condidtions of loading the incubator. With some incubators this might require forced draft circulation.

The participating laboratories shall periodically check and record the temperature attainment in the culture media and the uniformity of attainment throughout the incubator. The thermometers used for this purpose shall be checked for accuracy at the incubation temperature against a thermometer standardized by the Bureau of Standards. The thermometer scale should be subdivided to at least 0.2° C. preferably 0.1° C.

A. Procedure

1. Transfer a loopful (loop not less than 3mm diameter) of culture from the gas positive fermentation tube (Lauryl Tryptose Broth) to a fermentation tube of the liquid confirmatory medium (E.C. Medium).

Incubate the inoculated liquid confirmatory tubes in a water bath at 44.5° C ± 0.2° C. or in an air incubator at 45.5° C ± 0.2° C. The temperature stipulated shall be that of the culture medium.

2. Streak one or more plates of EMB agar with culture from the same gas positive presumptive tubes. Incubate the EMB at 35° C. ± 0.5° C. for 24 ± 2 hours.*

3. From each of the EMB plates streaked from the presumptive tubes fish two typical coliform colonies to lactose broth fermentation tubes. If not typical coliform colonies are present, fish two or more colonies considered most likely to consist of organisms of the coliform group. Incubate the lactose broth tubes at 35° C until gas formation is noted, the incubation not to exceed 48 hours.

Continue with the differentiation by IMViC procedure as outlined in item 3 above.

B. Expression of Results

Production of gas in the liquid confirmatory tube constitutes a Positive Partially Confirmed Test for E. coli. Demonstration of the presence of E. coli types 1 and 2 by the IMViC differentiation constitutes a Positive Completed Test.

MPN's shall be computed from the positive and negative tube pattern of the completed test results should include a statement of the type and temperature of incubation.

VI. Media and Glassware - Composition and Preparation

Procedures for the preparation of culture media and dilution water, and sterilization of glassware shall be in conformity with the methods and formulae described in "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes", Tenth Edition pp 362-373.

For the purpose of obtaining greater uniformity in results, however, the participants in the co-operative survey shall use dehydrated culture media exclusively.

*The bacteriological criteria for shucked oysters in the market recommended at the 1958 Shellfish Sanitation Workshop stipulates limitations based on MPN's derived from the E.C. partially confirmed test rather than from the completed IMViC test. In routine examinations of shucked eastern oysters at the market, therefore, only the partially confirmed test need be used.

LIQUID CONFIRMATORY PROCEDURE FOR THE ENUMERATION OF E. COLI

Gas Positive LST Tube

E.C. Medium Incubate in Water Bath 44.5°C. ± 0.2°C or Air Incubator 45.5°C. ± 0.2°C. Gas Positive EMB Agar Plates Incubate at 35°C. Lactose Broth Incubate at 35°C Purification of Culture IMViC	EMB Agar Plates Incubate at 35°C Lactose Broth Incubate at 35°C. Purification of Culture IMViC